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Efficient gene activation system on mammalian cell chromosomes using recombinant adenovirus producing Cre recombinase

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Abstract

To develop a method for activating genes located on cell chromosomes, an on/off switching unit regulated by the site-specific recombinase Cre was constructed. The switching unit was designed to express firstly the *neo* gene and secondly the reporter *lacZ* gene by Cre-mediated excisional deletion of the *neo* gene. CV1 cell lines bearing the switching unit on a cell chromosome were isolated and activation of the *lacZ* gene was examined after infection with a Cre-producing recombinant adenovirus. In one cell line virtually 100% of the cells stably expressed the *lacZ* gene, whereas in another cell line *lacZ*-expressing cell populations reached only to about 90% and decreased after cell divisions. The Southern blot analyses showed that the latter type of cells contained a head-to-tail array of the switching units, and that consequently the *lacZ*-expressing units were excised from a cell chromosome and present as extrachromosomal circular DNAs. These results showed that the system offers efficient activation of genes introduced into cell chromosomes and that the organization of the reporter units are important for efficiency and duration of the activated gene expression.

Keywords: Adenovirus vector; *loxP* site

1. Introduction

Methods of regulating the expression of genes introduced into mammalian cell chromosomes are important in many fields of basic research and in gene therapy. Cre recombinase (Sternberg and Hamilton, 1981) mediates precise site-specific excisional deletion of DNA flanked by a pair of its recognition sites, *loxP*. A Cre/*loxP* system has recently been successfully utilized in a gene activation and inactivation strategy in transgenic mice and ES cells (Lakso et al., 1992; Orban et al., 1992; Gu et al., 1993, 1994; Kuhn et al., 1995). However, applications of the Cre/*loxP* system to widely-used cultured cells is still very limited because only a small fraction of cells can be subjected to gene activation by Cre recombinase produced as a result of DNA transfection or electroporation (Sauer and Henderson, 1989; O'Gorman et al., 1991; Fukushige and Sauer, 1992).

Here we report that Cre-mediated gene activation can be achieved in virtually 100% of cultured cell population on cell chromosomes by using a replication-deficient recombinant adenovirus (Ad) expressing a modified cre gene (Kanegae et al., 1995) tagged with nuclear localization signal (NLS) (Kalderon et al., 1984). Therefore, the Cre-producing recombinant Ad offers an efficient gene-activation system which seems superior to that of current inducible promoters in a number of points. Moreover, it enables all sorts of gene manipulations mediated by the Cre recombinase to be efficiently performed in various cultured cell lines and probably in transgenic mice.

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Abbreviations: Ad, adenovirus; kb, kilobase(s); MOI, multiplicity of infection; NLS, nuclear localization signal; ONPG, o-nitrophenyl-β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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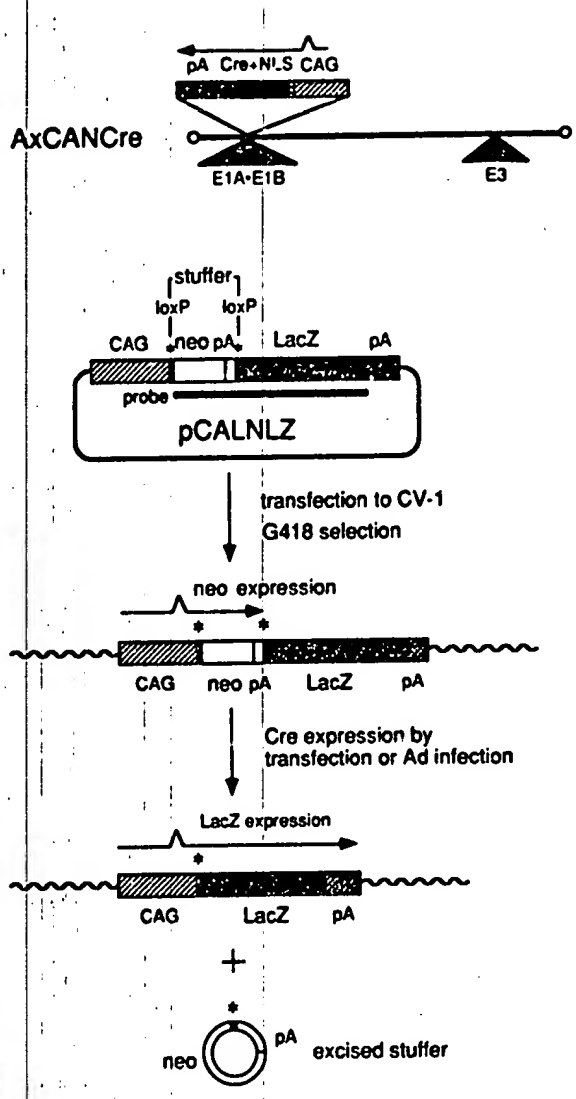


Fig. 1. Strategy for activation of the *lacZ* gene on a chromosome. (a) Structure of the Cre-producing recombinant Ad. CAG, CAG promoter; NLS, nuclear localization signal of SV40 T antigen (Graham et al., 1977); pA, polyadenylation signal. The filled triangle indicates deletion of Ad genes. (b) Structure of the gene activation unit and the activation strategy. Probe, the labeled probe used in Fig. 3a; wavy line, cell chromosomal DNA; *, *loxP* site. The plasmid pCALNLZ has been described in Kanegae et al., 1995. pCALNLw, a derivative from pCALNLZ with a cloning site instead of the *lacZ* gene, are also available on request. A recombinant Ad expressing a modified cre gene, AxCANCre, was described previously (Kanegae et al., 1995). It was constructed by cotransfection of 293 cells (Graham et al., 1977) with the cassette cosmid pAxCAwt containing the cre expression unit and Ad5-dlX DNA-terminal protein complex (COS-TPC method: Miyake et al., 1996).

2. Experimental and discussion

2.1. Gene activation strategy

The recombinant Ad AxCANCre (Kanegae et al., 1995) (Fig. 1a) efficiently produces an NLS-tagged Cre recombinase under control of the CAG promoter (Niwa et al., 1991). The strategy of gene activation on cell chromosomes is illustrated in Fig. 1b. The reporter, the CALNLZ switching unit (Kanegae et al., 1995) in plasmid pCALNLZ, consists of the CAG promoter, a stuffer, the *E. coli lacZ* gene and a polyadenylation (poly(A)) signal. The stuffer was composed of the *neo* gene and another poly(A) signal flanked by a pair of *loxP* sites, and was interposed between the CAG promoter and the *lacZ* gene so that *lacZ* expression was hampered by the termination of the mRNA sequence at the poly(A) site within the stuffer. Consequently, the reporter expression unit is expected initially to express the *neo* gene but not the *lacZ* gene. CV1 cells were transfected with pCALNLZ and *neo*-resistant cell clones were isolated. The *lacZ* gene of the CALNLZ switching unit located on the cell chromosome was expected to be activated by the Cre-mediated excisional deletion of the stuffer when a sufficient amount of the Cre recombinase was expressed through either transfection of a Cre-producing plasmid or infection by Cre-producing recombinant Ad.

2.2. *Activated* lacZ *expression*

Nineteen *neo*-resistant cell lines were isolated and none of them showed detectable staining by X-gal. Of these cell lines, 76% (14/19) showed activation of β-galactosidase production after AxCANCre infection (data not shown). Two representative cell lines, 2-7 and 2-2, were chosen because of their high β-galactosidase production and simple integration pattern by Southern analysis.

Activation of the *lacZ* gene in the cell clones 2-7 and 2-2 was assessed by X-gal staining (Fig. 2). No detectable staining was observed in mock-infected cells (panels c and f) or in mock-transfected cells (data not shown) in either cell line, suggesting that the background *lacZ*-expression from a potent CAG promoter was efficiently shut off by the stuffer. Three days after transfection with the Cre-producing plasmid, about 20% of the cells of both cell lines stained blue, showing that the expected activation of the *lacZ* gene had occurred in the stained cells (a and d). All of the 2-7 cells and about 90% of the 2-2 cells appeared to have stained with X-gal three days after AxCANCre infection at MOI 10 (b and e), indicating that the Cre-mediated gene activation occurred efficiently. The overall β-galactosidase activity of the Cre-producing Ad-infected cells was about 20-fold higher than that of the Cre-producing plasmid-transfected cells in either cell line (Table 1).

Significant differences in activated *lacZ*-expression were observed in these two cell lines. All of the 2-7 cells appeared to stain when infected with AxCANCre at MOI > 3, suggesting that the expected Cre-mediated recombination had occurred in every cell. Surprisingly, the stained population of 2-2 cells never reached 100% (remaining at about 90%) even when infected at MOI 50, and the cells always stained with various degrees of

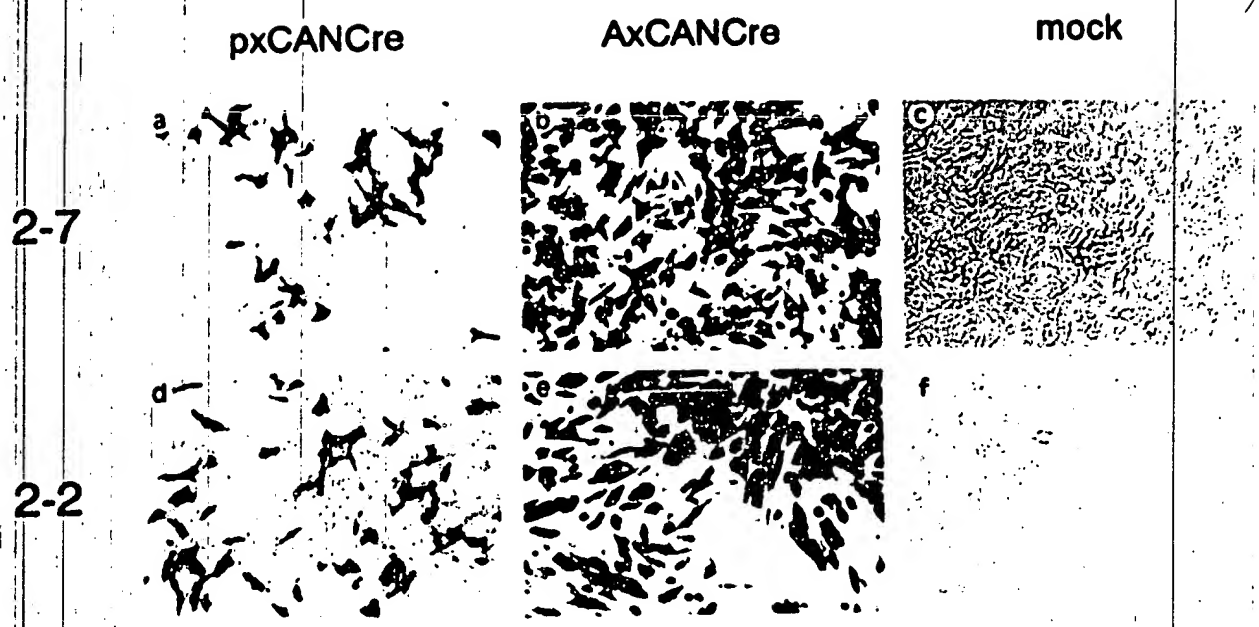


Fig. 2. Gene activation of the *lacZ* expression unit located on a cell chromosome. Cell clone 2-7 (a–c) and 2-2 (d–f) were either transfected with the plasmid pxCANCre containing the cre expression unit identical to AxCANCre (10 μg per 6 cm dish) (a and d), infected with AxCANCre at MOI 10 (b and e), or mock-infected (c and f). Three days later, the cells were stained with X-gal. **Methods:** To isolate 2-7 and 2-2 cells, gene-activation plasmid pCALNLZ DNA (Kanegae et al., 1995) was introduced into a CV1 chromosome by calcium-phosphate transfection (CellPhect transfection kit, Pharmacia) and subsequent G418 selection with 1.0 mg/ml of G418. Cells expressing *lacZ* gene after cre expression is conveniently identified by staining an aliquot of cells with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) after culturing them with medium containing 10^7 plaque-forming units/ml of AxCANCre virus for three days. Cell lines containing pCALNLZ DNA at a single site in a CV1 chromosome were selected by Southern blot analysis. For cell staining, 3 days after infection of AxCANCre at multiplicity of infection (MOI) 10, cells were washed with phosphate-buffered saline twice, fixed with 0.25% glutaraldehyde and stained with 0.1% X-gal.

Table 1
β-Galactosidase activity expressed in 2-7 and 2-2 cells[a]

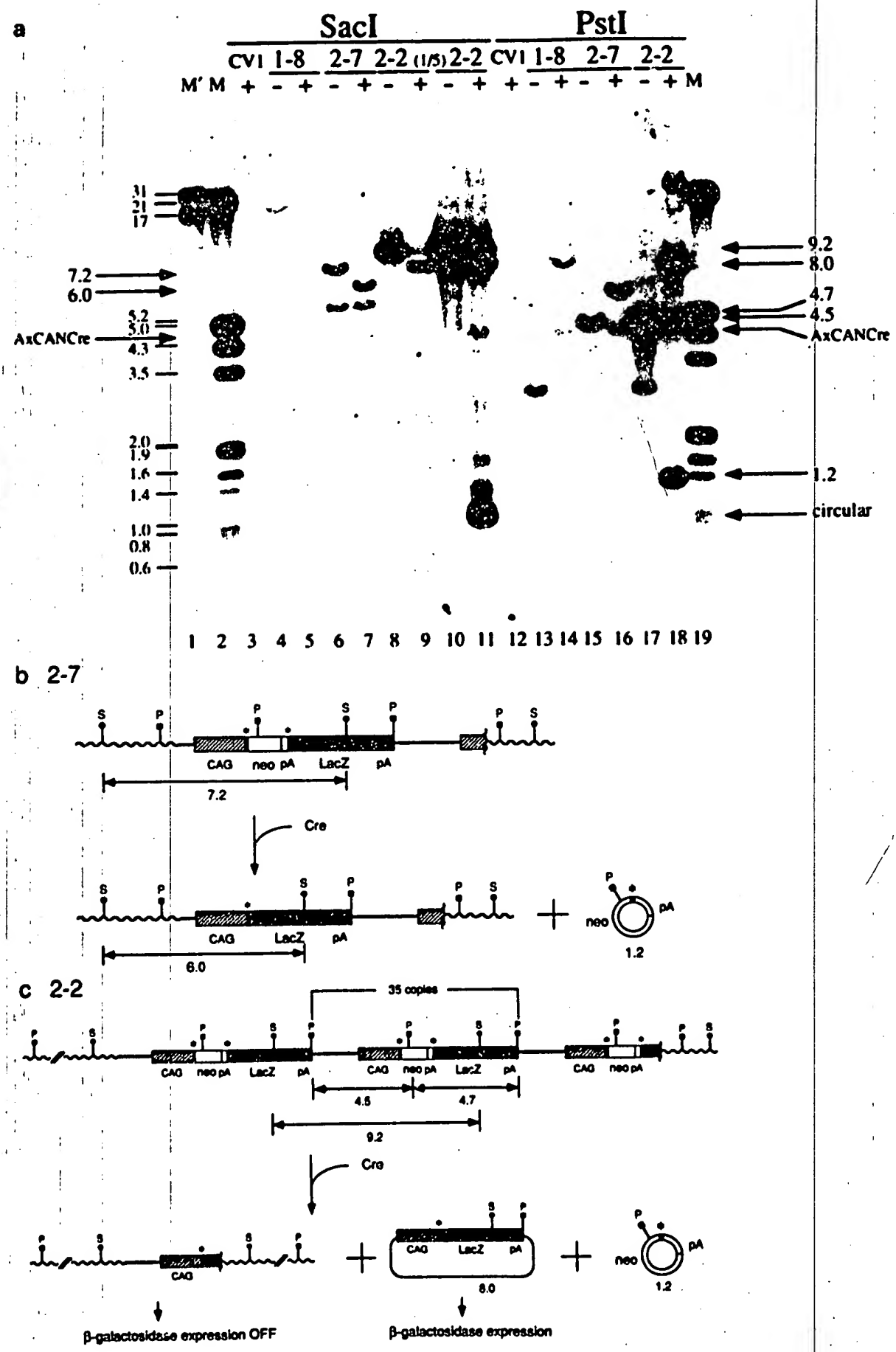
Cell line	β-Galactosidase activity (nanounits/cell)		
	Transfection pxCANCre	Infection AxCANCre	Mock infected
2-7	3.3	60.4	0.0
2-2	7.5	155.3	0.0

[a]To quantify β-galactosidase activity, 3×10^5 cells were infected with each of recombinant Ad at MOI 10 or transfect with 10 μg of plasmid pxCANCre, which contains the cre-expressing unit identical to that of AxCANCre. The cells were collected by centrifugation and suspended in 20 mM phosphate buffer (pH 7.2), 50 mM β-mercaptoethanol and 1 mM $MgCl_2$. The cells were then disrupted by sonication and an equal volume of 80% glycerol was added. After removing cell debris by centrifugation at 15 krpm for 10 min, the supernatant was subjected to the color reaction with o-nitrophenyl-β-D-garactopyranoside (ONPG).

intensity (typically in Fig. 2e). These observations were explained later (see Section 2.3)) by the analyses of the integrated reporter units.

2.3. Southern blotting analysis

To obtain direct evidence that the expected Cre-mediated recombination occurred in the AxCANCre-infected cells, the structures of integrated reporter units were analyzed by the Southern technique (Fig. 3a) and the structures of the integrated DNA of cell line 2-7 and 2-2 could be deduced. The results of 2-7 cells (Fig. 3a, lanes 6 and 15) suggested that 2-7 cells contain a single copy of intact CALNLZ unit on a cell chromosome (Fig. 3b, upper panel). After infection with the Cre-producing Ad, the stuffer-containing 7.2-kb band of the *Sac*I digest (lane 6) had shifted to the 6.0-kb band and was not seen in its original position (lane 7), suggesting that the expected excision of the 1.2-kb stuffer had occurred in almost all of the cells. Southern analyses of 2-2 cell DNA (lanes 8, 10 and 17) suggested that the cells contained about 35 copies of the pCALNLZ plasmid in a head-to-tail tandem array integrated at a single site on a cell chromosome (Fig. 3c; data of quantitative Southern analysis not shown). After infection with Cre-producing Ad, the tandem array yielded not only the 1.2-kb circular stuffer DNA (Fig. 3c, lower right and Fig. 3a, lane 11; a linearized form was seen in lane 18) but also the remaining *lacZ*-expression unit in the form of a 8.0-kb circle (Fig. 3c, lower middle). The closed circular form of the 8.0-kb DNA was confirmed using DNA digested with *Eco*T22I, which does not cut pCALNLZ (data not shown). The generated circular 8.0-kb DNA should express *lacZ*, because the *lacZ* coding region has been connected directly with a CAG promoter.


a
SacI
PstI
CV1 1-8 2-7 2-2 (1/5) 2-2 CV1 1-8 2-7 2-2
M' M + - + - + - + - + + - + - + - + M
31
21
17
7.2
6.0
5.2
5.0
AxCANCre
4.3
3.5
2.0
1.9
1.6
1.4
1.0
0.8
0.6
9.2
8.0
4.7
4.5
AxCANCre
1.2
circular
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
b 2-7
S P P S P P S
CAG neo pA LacZ pA
7.2
Cre
CAG LacZ pA
neo pA
6.0
1.2
c 2-2
35 copies
CAG neo pA LacZ pA
4.5
4.7
9.2
Cre
CAG
CAG LacZ pA
neo pA
8.0
1.2
β-galactosidase expression OFF
β-galactosidase expression

Judging from the intensity of the weak 9.2-kb unprocessed band in the *Sac*I digest (lane 9, compare with lane 8), the overall processing efficiency of the 35-copy reporter units in 2-2 cells reached about 90%. The processing efficiency on the cell chromosome is much higher than those by using transfection or electroporation in previous reports (O'Gorman et al., 1991; Fukushige and Sauer, 1992). If all the DNA interposed among the approximately 70 *loxP* sites in 2-2 cells had been completely excised, the leftmost and rightmost cell-plasmid junctions should be connected at a single *loxP* site and form a single DNA fragment located on the cell chromosome (Fig. 3c, lower left). Such DNA fragments detected in 2-2 cells were incapable of expressing the *lacZ* gene, because the *lacZ* gene was truncated by an integration junction with the cell chromosome.

The structure of the integrated reporter units described above explains well the difference between *lacZ* expression in 2-7 and 2-2 cells. After switching on expression, the 2-7 cells expressed the *lacZ* gene uniformly with apparently 100% efficiency, and stably, because there was only one copy and the activated expression unit is located on a cell chromosome. In contrast, 2-2 cells expressed the *lacZ* gene to various extents, some cells did not stain in the three-day period, and *lacZ* expression decreased day by day (data not shown). These results can be explained because the activated expression units were present in the extrachromosomal and multicopy state. Since most of the units were processed within several hours after infection (data not shown), some of the cells may have lost most of their copies of the extrachromosomal expression units by chance after two or three cycles of cell division. These findings suggest that two types of cell line can be obtained after introduction of a switching unit into the cell chromosome via transfection and drug selection. The critical difference is whether the remaining integrated copy after Cre exposure can express the desired gene or not.

Additional three highly-expressing cell lines were examined by Southern analysis. All three of them contained multiple copies of the target plasmid and some of the copies were present in a head-to-tail tandem array (data not shown). The results suggest that, by the transfection condition used here, most of highly-expressed cell lines contained multiple copies of the target plasmid and therefore show mixed phenotype of 2-7 and 2-2. Cell lines like 2-7 are desirable for most purposes because the activated expression is stable, and for that reason cell lines like 2-2 or of the mixed phenotype should be avoided.

Very recently, Sakai et al. (1995) reported a similar gene activation system using recombinant Ad producing the authentic Cre recombinase but they did not aware of the practically important point that two different types of cell line have to be generated in this method. The recombinant Ad producing NLS-tagged Cre (AxCANCre) was three-fold more active than a recombinant Ad producing the authentic Cre (AxCACre) judged from serial-infection/staining experiments using cell line 2-7 (data not shown). This experiments also suggested that the cell line 2-7 is useful for measuring the Cre activity in mammalian cells.

3. Conclusions

(1) We have shown that the transgene introduced into cell chromosomes can be manipulated efficiently in individual cells by using a Cre-producing Ad. The strategy using Cre-producing Ad seems superior to the current gene regulation system using inducible promoters. There are three advantages of gene activation by this method: (i) The level of background expression before switching-on is very low, (ii) a very potent versatile promoter can be used after switching-on, and (iii) the switching-on state is stable after cell divisions. The Cre could be produced by retrovirus vectors but Ad vector seems superior mainly because (i) the expression efficiency is much higher, and (ii) the cre gene is eliminated from the cells spontaneously.

Fig. 3. Structure of the activated expression unit on a cell chromosome. (a) Southern blot analysis of three different cell lines (1-8, 2-7 and 2-2) before (−) and after (+) AxCANCre infection. The probe used is shown in Fig. 1. Cell line 1-8 contains a partial single copy of the reporter unit truncated in the middle of *lacZ* gene (data of X-gal staining and a deduced structure are not shown). CV1(+) (lanes 3 and 12) is the parent cell line infected with AxCANCre. In the 2-2(1/5) lanes (lanes 8 and 9), one-fifth (1 μg/lane) of 2-2 DNA was used while 5 μg of DNA sample were used in the other lanes including lanes 2-2 (lanes 10 and 11). Lambda-DNA samples digested with *Kpn*I (M′) and *Eco*RI + *Hind*III (M) were used as size markers. The size of thick, multicopy bands derived from 2-2 cells are indicated by long arrows on the right side of the gel. The arrow labeled 'circular' points to the band of the closed-circular form of the excised stuffer in 2-2 cells (lane 11). The arrow labeled 'AxCANCre' points to faint bands derived from infected AxCANCre DNA because of short polylinker homology with the probe. The bands of *Sac*I digest containing the 1.2-kb stuffer sequences in the (−) lanes (lanes 4, 6 and 8) shifted to 1.2-kb lower bands in the corresponding (+) lanes (lanes 5, 7 and 9). Because the *Pst*I site within the stuffer was deleted by Cre-mediated stuffer excision, the bands of *Pst*I digest containing the stuffer sequences in (−) lanes (lanes 13, 15 and 17) shifted to longer bands in the corresponding lanes (+) (lanes 14, 16 and 18). (b) and (c) Deduced structure of integrated pCALNLZ DNA in cell lines 2-7 and 2-2, respectively. S, *Sac*I site; P, *Pst*I site; ▼, *loxP*. Methods: Cells in 6-cm dishes were infected with AxCANCre at MOI 10. Total cell DNAs prepared by the standard procedure (Maniatis et al., 1982; Sambrook et al., 1989) were digested with the restriction enzymes (5 μg per lane) and were then subjected on a 1% agarose gel. Electrophoresis was performed for 16 h at 35 V. The probe DNA (shown in Fig. 1b; the *Pst*I-*Eco*RI fragment containing *neo*, *loxP* and *lacZ* genes) was labeled with digoxigenin-UTP and the specific DNA was detected by autoradiography for 4 h with CSPD chemiluminescence (Boeringer).

(2) The advantages of this system enable us to establish cell lines containing inactivated genes whose high expression would be deleterious to cell growth or cell cycling. The function of such genes can be investigated after switching-on in a time-dependent manner. For example, genes related with cell cycle, carcinogenesis, apoptosis and cell differentiation seems particularly useful. Development of efficient packaging cell lines of gene-therapy vectors is another potential application. Because the recombinant Ad can be administered to animals, positional knock-out and switching-on can be considered in transgenic animals.

(3) It is practically important to note that two types of cell line will be obtained after G418 selection. The expressing *lacZ* gene is located on a chromosome in one type and on excised circular DNAs in the other type. The latter type of cell lines should be avoided when a long-term expression is desired after the gene activation.

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